Escherichia coli dihydrodipicolinate synthase and dihydrodipicolinate reductase: kinetic and inhibition studies of two putative herbicide targets

Carolyn V Coulter,[1] Juliet A Gerrard,[2,3]* James AE Kraunsoe[3] and Andrew J Pratt[1,3]

[1]*Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch, New Zealand*
[2]*Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand*
[3]*Dyson Perrins Laboratory, South Parks Road, Oxford, OX1 3QY, UK*

Abstract: Dihydrodipicolinate synthase (DHDPS) (EC 4.2.1.52) and dihydrodipicolinate reductase (DHDPR) (EC 1.3.1.26) have attracted much recent attention as potential herbicide targets. DHDPS was feedback-inhibited by (*S*)-lysine; inhibition was reversible and uncompetitive with respect to both (*S*)-ASA and pyruvate. Homoserine lactone was a reversible non-competitive inhibitor of DHDPS with respect to both (*S*)-ASA and pyruvate. (*R*)-Cysteine sulfinic acid and (*S*)-glutamic acid were reversible uncompetitive inhibitors of DHDPS with respect to (*S*)-ASA. (*S*)-Aspartic acid was a reversible mixed-type inhibitor. Dipicolinic acid was a reversible competitive inhibitor of DHDPR with respect to the substrate (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid, as was isophthalic acid. Δ^3-Tetrahydroisophthalic acid was a moderate inhibitor of both DHDPS and DHDPR. These compounds represent possible leads in the development of novel herbicides.

Keywords: dihydrodipicolinate synthase; dihydrodipicolinate reductase; lysine biosynthesis; homoserine lactone

1 INTRODUCTION

(*S*)-Lysine is an essential amino acid. Thus, inhibitors of the pathways of (*S*)-lysine biosynthesis are of interest as they may be toxic to plants and microorganisms, but not to animals. Such inhibitors are, therefore, potential herbicides and/or bacteriocides. While several classes of herbicides and bacteriocides have been found to inhibit amino acid biosynthesis, there are, as yet, no commercially significant examples of such agents which act by interfering with the (*S*)-lysine biosynthetic pathway. The enzymes of (*S*)-lysine biosynthesis are, therefore, attracting increasing attention in the literature.[1] In addition, since (*S*)-lysine is the limiting amino acid in many cereal crops,[2,3] an understanding of (*S*)-lysine biosynthesis may lead to more nutritious crops in the future.

(*S*)-Lysine is synthesised via the diaminopimelate (dap) pathway in bacteria and plants.[1] The genes of the enzymes of the dap pathway have been well mapped in *E coli*, providing a basis for the study of these enzymes.[4] We are involved in a study of the first two committed steps in the dap pathway of (*S*)-lysine biosynthesis: the condensation of (*S*)-ASA with pyruvate to form an unstable heterocycle, now thought to be (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinate,[5] catalysed by the enzyme dihydrodipicolinate synthase (DHDPS); and the reduction of this product to yield tetrahydrodipicolinate, catalysed by dihydropicolinate reductase (DHDPR), with concomitant oxidation of NAD(P)H[6] (Fig 1). DHDPS is feedback-inhibited by the final product of the pathway, (*S*)-lysine, and, as such, is a key point in the regulation of (*S*)-lysine biosynthesis.[7,8] DHDPS has been isolated from *Escherichia coli*, as well as from other bacteria and several plant sources.[1]

Previous work in our group has studied the solution structure of (*S*)-aspartate β-semialdehyde (*S*)-ASA] using NMR techniques.[9] We question whether DHDPS recognises a straight-chain aldehyde or hydrate, or cyclic lactol (Fig 2). We report herein detailed evaluation of analogues of such a cyclic lactol structure as possible inhibitors of DHDPS, providing information about the physiologically relevant form of (*S*)-ASA and mechanistic insight useful for herbicide

* Correspondence to: Juliet A Gerrard, Department of Plant and Microbial Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand
E-mail: j.gerrard@botn.canterbury.ac.nz
Contract/grant sponsor: Shell Research Ltd
(*Received 6 September 1998; revised version received 15 April 1999; accepted 20 May 1999*)

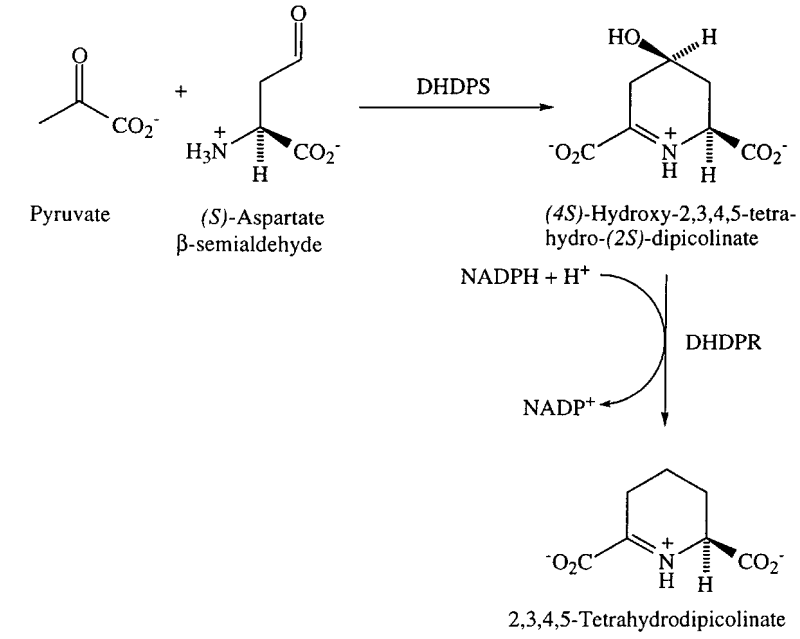

Figure 1. The enzyme-catalysed reactions of DHDPS and DHDPR.

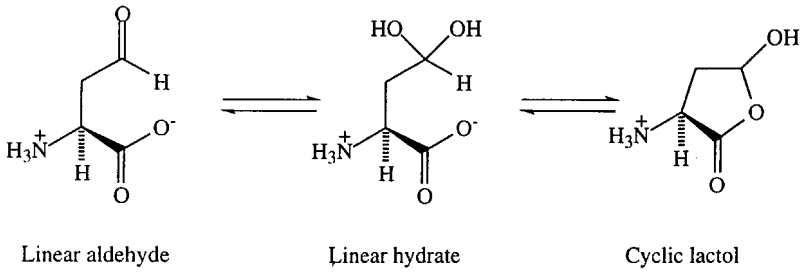

Figure 2. Possible structures of (*S*)-ASA.

design. We also report the evaluation of a variety of substrate analogues based on the straight-chain hydrate form of (*S*)-ASA as inhibitors of DHDPS, and a variety of analogues of dihydrodipicolinate as product inhibitors of DHDPS and substrate inhibitors of DHDPR. These new inhibitors may act as lead compounds for herbicide and bacteriocide research.

2 EXPERIMENTAL

2.1 Materials and methods

E coli XL-1 Blue was a gift from Jane Lancaster (Crop and Food Research Ltd, Christchurch, New Zealand) and both the *dap A* gene (encoding DHDPS) and the *dap B* gene (encoding DHDPR) present on the plasmid pBR322 were gifts from Dr C Richaud (Institut Pasteur, Paris, France). Pure (*S*)-ASA was synthesised either by the method of Coulter *et al*,[9] or a modification of the method by Tudor *et al*,[10] in which oxidation of diprotected (*S*)-allylglycine was mediated by osmium tetroxide and periodate rather than ozone. Unless otherwise stated, all chemicals were purchased from Aldrich. *o*-Aminobenzaldehyde was synthesised from *o*-nitrobenzaldehyde,[11] 3-aminopyrrolid-2-one was synthesised via a cyclisation on ethyl $\alpha\gamma$-diaminobutyrate dihydrochloride,[12] 2-aminocyclopentanone was synthesised from aminocyclopentane,[13] the sulfoxide[14] and sulfone[15] of (*S*)-methyl-(*R*)-cysteine were synthesised from (*S*)-methyl-(*R*)-cysteine; Δ^3-tetrahydroisophthalic acid was synthesised by the reduction of isophthalic acid,[16] and Δ^2-tetrahydroisophthalic acid was synthesised from 1,3-dibromopropane and diethyl malonate.[17]

Electrophoresis was performed using the method of Dunn,[18] a 4% stacking gel and a 12.5% resolving gel were used, with a constant current of 30 mA through the stacking gel and 40 mA through the resolving gel. The protein was visualised by staining with Coomassie Brilliant Blue.

2.2 Enzyme purification

All manipulations were carried out between 0 and 4 °C.

2.2.1 DHDPS

Preparation of a crude cell-free extract. DHDPS was prepared using *E coli* XL-1 Blue that had been transformed with pJG001 (derived from the multicopy plasmid pBluescript), which contains the *dap A* gene,[19] via the calcium chloride method. *E coli* XL-1 Blue pJG001 was cultured in 10 litres of LB broth; the cells were chilled on ice for 30 min, then harvested by

centrifugation (3000*g*, 10 min) to give 50 g wet weight of cells. Cells were washed in Tris HCl (20 mM, pH 8.0; 30 ml) then centrifuged (3000*g*, 10 min). The cell pellet was resuspended in an equal volume of Tris HCl (20 mM, pH 8.0) and flash-frozen in liquid nitrogen, followed by slowly thawing on ice at 4 °C overnight. This cycle was repeated seven times. After centrifugation (3000*g*, 10 min) the supernatant was collected (70 ml). The crude supernatant (35 ml) was loaded on to a Q-Sepharose ion exchange column (bed volume 70 ml, 15 × 2.6 cm, pre-equilibrated with Tris HCl (20 mM, pH 8.0)). The DHDPS was then eluted with a 0 to 1 M sodium chloride gradient in Tris HCl (20 mM, pH 8.0) at 4 °C. The eluted fractions were tested for DHDPS activity using the *o*-aminobenzaldehyde assay.[20] Active fractions eluted between 0.4 and 0.6 M sodium chloride, and were pooled and dialysed overnight in dialysis buffer [Tris HCl (20 mM, pH 8.0), EDTA (1 mM), 2-mercaptoethanol (1 mM), ammonium sulfate (10 g litre^{-1}), (2 × 2 litres)]. The dialysed sample then underwent further ion exchange chromatography. In preparation for kinetic studies, 1.0-ml batches of dialysed sample were charged onto a 1-ml Resource Q-Sepharose column (Pharmacia) equilibrated with Tris HCl (20 mM pH 8.0). The column was then eluted with a linear gradient of 0 to 1 M sodium chloride in Tris HCl (20 mM, pH 8.0). DHDPS eluted between 0.4 and 0.6 M sodium chloride and active fractions were pooled.

2.2.2 DHDPR

DHDPR was prepared using *E coli* XL-1 Blue, transformed with pJK001 (derived from pBluescript) which contains the *dap B* gene.[21] *E coli* XL-1 Blue pJK001 was cultured in LB broth, the cells were chilled on ice for 30 min, then harvested by centrifugation (3000*g*, 10 min), washed in Tris HCl (20 mM, pH 8.0; 30 ml), then recentrifuged (3000*g*, 10 min). The cell pellet was resuspended in an equal volume of Tris HCl (20 mM, pH 8.0) and ultrasonicated on ice, at 4 microns, for 4 min in 15-s bursts, with 15 s between each burst. The supernatant was then collected after centrifugation (3000*g*, 10 min). Aliquots (1.0 ml) were heat treated at 70 °C for 3 min, followed immediately by cooling on ice. The precipitated proteins were removed by centrifugation (3000*g*, 10 min), and the supernatant was collected. Solid ammonium sulfate was added to the supernatant until 20% saturation was achieved and stirring was continued for 30 min. The mixture was centrifuged (10 000*g*, 10 min) and further ammonium sulfate was added to the supernatant, until 60% saturation was achieved; stirring was continued for 30 min. The second precipitate was dissolved in a volume of Tris HCl (20 mM, pH 8.0) equal to the volume of solid. The ammonium sulfate precipitate was dialysed against 50 volumes of dialysis buffer overnight at 4 °C. The dialysed sample was loaded onto a Q-Sepharose ion exchange column (bed volume 70 ml, 15 × 2.6 cm) that had been pre-equilibrated with Tris HCl (20 mM, pH 8.0). The DHDPS was then eluted with a 0 to 1.0 M sodium chloride gradient in Tris HCl (20 mM, pH 8.0), active fractions eluted between 0.6 and 0.9 M sodium chloride and were pooled. A DHDPR solution of concentration greater than 0.7 mg ml^{-1} was prepared for electrospray mass spectrometry by concentration against dry Sephadex. A volume of 10 µl was injected into the electrospray source via a loop injector (Rheodyne 5717) as a solution, 25 M in water + acetonitrile (1+1 by volume) plus 10 ml litre^{-1} formic acid, at a flow rate of 2 µl min^{-1}. The mass spectrometer was scanned over the mass range 900–1900 Da. The instrument was calibrated with myoglobin.

2.3 Enzyme assays

Unless otherwise stated, the standard assay for DHDPS activity was performed in MOPS buffer, pH 7.2 (final concentration 100 mM), containing NADPH (0.162 mM) DHDPS (4.8×10^{-4} mg), DHDPR (approximately 10-fold excess), (*S*)-ASA, and pyruvate. DHDPR was judged to be in excess when addition of further DHDPR did not result in an increased rate of reaction; analogous considerations were applied to the manipulation of (*S*)-ASA, and pyruvate concentrations. When the kinetic parameters for (*S*)-ASA were determined, concentrations were varied between 0.06 and 0.25 mM and pyruvate was present in excess at a concentration of 40 mM, while the kinetic parameters for pyruvate were determined by varying pyruvate concentrations between 0.05 to 0.5 mM, with (*S*)-ASA present in excess at 2.5 mM.

The assay mixture was incubated at 30 °C; the reaction was initiated by the addition of the substrate (*S*)-ASA with thorough mixing. The absorbance at 340 nm was followed over 300 s, blanked against distilled water, where utilisation of NADPH corresponded to the utilisation of substrate. All kinetics measurements were performed in duplicate, and all experiments were repeated several times to ensure that reproducible K_m and V_{max} values were obtained. Duplicate measurements were typically no more than 15% different.

The standard assay for DHDPR was modified from that above for DHDPS: MOPS buffer, pH 7.2 (final concentration 100 mM), containing NADPH or NADH, DHDPR (1.0×10^{-4} mg), DHDPS (approximately 10-fold excess), *(S)*-ASA, and 40 mM pyruvate. DHDPS was judged to be in excess when addition of further DHDPS did not result in an increased rate of reaction; analogous considerations were applied to manipulations of *(S)*-ASA, NADPH, and NADH concentrations. When the kinetic parameters for the substrate were determined, *(S)*-ASA concentrations were varied between 0.05 and 0.30 mM and NADPH was present at 0.162 mM; kinetic parameters for NADPH and NADH were determined by varying the co-factor concentration between 0.01 and 0.04 mM (for NADPH) and between 0.004 and 0.032 mM (for NADH), with the substrate present in excess at 2.5 mM. The assay was initiated by the

Purification step	Total protein ($mg\,ml^{-1}$)	Specific activity ($\mu mol\,s^{-1}\,mg^{-1}$)	Recovery (%)	Purification (-fold)
Crude supernatant	10.2	1.70×10^{-2}	100	–
First ion exchange + dialysis	1.6	3.24×10^{-2}	85.5	1.9
Second ion exchange	4.0×10^{-2}	4.70×10^{-1}	9.7	27.6

Table 1. Purification of DHDPS

addition of the DHDPR, where the *(S)*-ASA had been pre-equilibrated with the DHDPS for 5 min.

3 RESULTS AND DISCUSSION

3.1 Purification of DHDPS and DHDPR

DHDPS was extracted from *E coli* XL-Blue containing pJG001, a plasmid which over-expressed the enzyme several hundred-fold. Freeze thawing proved to be the simplest method for selective release of the enzyme from the cells, with seven cycles optimising specific activity. Purification of the crude extract (Table 1) was achieved using modifications of the methods of Yugari and Gilvarg,[22] and Shedlarski.[23] SDS-PAGE performed on the crude extract revealed that a protein with a molecular mass of ~31 kDa was the major component; this corresponded to DHDPS. An alternative purification of *E coli* DHDPS, yielding crystalline material,[24] is clearly more complete. However, the purification detailed here is a convenient and simple method for obtaining a homogeneous solution of DHDPS, appropriate for kinetic studies. The final specific activity of DHDPS was 4.7×10^{-1} μmol $s^{-1}mg^{-1}$ of protein, and monomeric protein was judged homogeneous by SDS-PAGE stained with Coomassie Brilliant Blue.

DHDPR was extracted from *E coli* XL-Blue containing pJK001, a plasmid that over-expressed the enzyme several hundred-fold.[21] DHDPR was purified to a specific activity of 2.25 μmol $s^{-1}mg^{-1}$ of protein, using a method based on the method of Tamir and Gilvarg[25] (Table 2). The purified enzyme was homogeneous as judged by SDS-PAGE, stained with Coomassie Brilliant Blue. The electrophoretic mobility suggested monomeric protein of molecular mass ~29 kDa. Electrospray mass spectrometry was performed on DHDPR, yielding a molecular mass of 28 762 (±5) Da for the monomer. This is in good agreement with the literature value[27] of 28 758 (±8), and the value calculated from the gene sequence (28 757).

3.2 Choice of assay

In order to study the kinetic properties of the two enzymes DHDPS and DHDPR, a quantitative assay was required. For DHDPS there are three assays in the literature: the imidazole buffer assay, the *o*-aminobenzaldehyde assay, and a coupled assay.[20]

The imidazole buffer assay involves monitoring a rise in absorption at 270 nm on incubation of the DHDPS enzyme and its substrates (*(S)*-ASA and pyruvate) in imidazole buffer. However, the exact nature of the chromophore formed is unknown and a lag phase is present before the absorbance at 270 nm increases. Thus, it is questionable whether the rate measured is actually the initial rate of the DHDPS-catalysed reaction. However, it is easy to perform and continues to be used by other workers.[24]

The addition of *o*-aminobenzaldehyde to a solution containing the DHDPS-catalysed reaction immediately results in a yellow solution that slowly develops a deep purple chromophore; this colour development is accelerated by acid treatment. This assay is an extremely useful qualitative tool, as it is both highly specific and extremely sensitive. It was therefore the method of choice for DHDPS purification. Again, the nature of the chromophore has yet to be determined. It has been hypothesised that the product of the enzymatic reaction is oxidised to dipicolinate, which then reacts with *o*-aminobenzaldehyde to form the purple chromophore.[28] However, addition of *o*-aminobenzaldehyde to a dipicolinic acid solution, under the conditions of the assay, did not result in the formation of a purple adduct. This result would appear to invalidate the hypothesis, and the nature of the purple chromophore remains unclear.

The DHDPS-DHDPR coupled assay involves following the activity of DHDPS by monitoring, at 340 nm, the utilisation of NADPH by DHDPR. This

Purification step	Total protein ($mg\,ml^{-1}$)	Specific activity ($\mu mol\,s^{-1}\,mg^{-1}$)	Recovery[a] (%)	Purification[a] (-fold)
Crude supernatant	9.0	7.89×10^{-4}	100	–
Heat treatment	3.4	1.14×10^{-4}	5.4	0.14
Ammonium sulfate precipitation + dialysis	3.0	4.45×10^{-4}	18.8	0.56
Ion exchange	1.0×10^{-2}	2.25	10	2850

[a] Crude preparations of DHDPR contain DHDPS and other enzymes that consume NADPH and interfere with the coupled assay; the numbers in this column are therefore not true measures of purification.

Table 2. Purification of DHDPR

Table 3. Kinetic parameters of DHDPS and DHDPR[a]

Enzyme	*Substrate*	K_m (10^{-4} M)	V_{max} (μmol s^{-1} mg^{-1})
DHDPS	(*S*)-ASA	1.38	0.723
	pyruvate	1.34	0.549
DHDPR	HTHDPA	5.29	0.571
	NADPH	0.165	0.363
	NADH	6.56×10^{-2}	0.236

[a] Values were calculated using a direct linear plot.

assay is able to measure DHDPS kinetics, if DHDPR is present in excess, and DHDPR kinetics, if DHDPS is present in excess. Although the coupled assay was originally used by Yugari and Gilvarg in 1965, it has been little used until recently. With the ease of obtaining large (milligram) quantities of recombinant DHDPS and DHDPR, there has been a resurgence in the use of this assay[19,29] which was therefore selected, with slight modification, for detailed kinetic studies.

3.3 Measurement of kinetic parameters for DHDPS and DHDPR

The kinetics of the reaction were thoroughly investigated, and the results were interpreted in terms of the Michaelis–Menten model, analysing initial rates of reaction by Lineweaver–Burk (1/Rate versus 1/[Substrate]), Eadie Hofstee (Rate versus Rate/[Substrate]), and direct linear (Rate versus -[Substrate]) plots (Table 3). All graphical methods used were in close agreement. For DHDPS and *(S)*-ASA, these values are similar to those obtained by other groups using the coupled assay, for example K_m 1.3×10^{-4} M[22] and K_m 1.7 $(\pm 0.1) \times 10^{-4}$ M.[29] However, they are considerably lower than those obtained using the imidazole buffer assay, for example K_m 2.3×10^{-4} M[28] and K_m 5.5×10^{-4} M.[24] For pyruvate, our values were again much closer to those obtained using the coupled assay, for example K_m 2.5×10^{-4} M[22] and K_m 1.7 $(\pm 0.3) \times 10^{-4}$ M,[29] than those using the imidazole buffer assay, for example K_m 5.7×10^{-4} M.[24]

The kinetic parameters of DHDPR with respect to its substrate, *(4S)*-4-hydroxy-2,3,4,5-tetrahydro-*(2S)*-dipicolinate (Fig 3), and the two possible cofactors, NADPH and NADH, were also measured (Table 3). DHDPR is an unusual enzyme in that it can utilise NADPH or NADH; most nicotinamide-dependent enzymes show a marked preference for one or other of these two cofactors. NADPH is usually the cofactor of choice in the coupled assay due to its greater chemical stability. Our values were comparable to the literature values.[25–27] The low K_m reflects the high affinity the enzyme has for its cofactor.

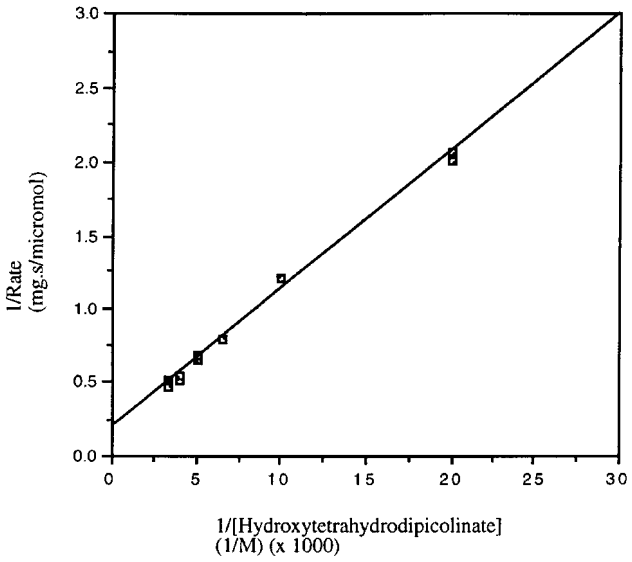

Figure 3. Lineweaver–Burk plot of the kinetics of DHDPR with respect to (*4S*)-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinate.

3.4 Inhibition of DHDPS and DHDPR

Detailed inhibition kinetics were run at a range of inhibitor and substrate concentrations, and analysed using Dixon (1/Rate versus [Inhibitor]) and modified Dixon ([Substrate]/Rate versus [Inhibitor]) plots[30] to establish the mode of inhibition. K_i values are listed in Table 4. No inhibition of *E coli* DHDPS was observed by high concentrations, up to 10 mM, of the substrate *(S)*-ASA. This is in contrast to the results of other workers using the same assay.[28] This difference may be attributable to impurities present in the substrate preparation in the earlier study. *(S)*-Lysine was found to inhibit the enzyme. Inhibition of DHDPS by *(S)*-lysine was shown to be uncompetitive with respect to both *(S)*-ASA and pyruvate. These results suggest that the *(S)*-lysine binding site is allosteric, at a site remote

Table 4. K_i and K'_i values for inhibition of DHDPS and DHDPR[a]

Enzyme	*Substrate*	*Inhibitor*	K_i (M)	K'_i (M)	*Mode of inhibition*
DHDPS	(*S*)-ASA	HSL	$1.2–2.2 \times 10^{-2}$	$= K_i$	Non-competitive
	Pyruvate		$0.8–1.5 \times 10^{-2}$	$= K_i$	Non-competitive
	(*S*)-ASA	2-Aminocyclopentanone	$1.2–2.4 \times 10^{-2}$	$= K_i$	Non-competitive
		(*R*)-cysteine sulfinic acid	–	$6.1–8.6 \times 10^{-3}$	Uncompetitive
		(*S*)-glutamic acid	–	$0.9–1.4 \times 10^{-2}$	Uncompetitive
		(*S*)-aspartic acid	$0.9–1.4 \times 10^{-4}$	$2.1–3.9 \times 10^{-2}$	Mixed
DHDPR	HTHDPA	dipicolinic acid	$4.3–4.4 \times 10^{-4}$	–	Competitive
		isophthalic acid	$5.4–5.6 \times 10^{-3}$	–	Competitive

[a] All inhibition was reversible. Values were calculated from Dixon and modified Dixon plots, respectively.

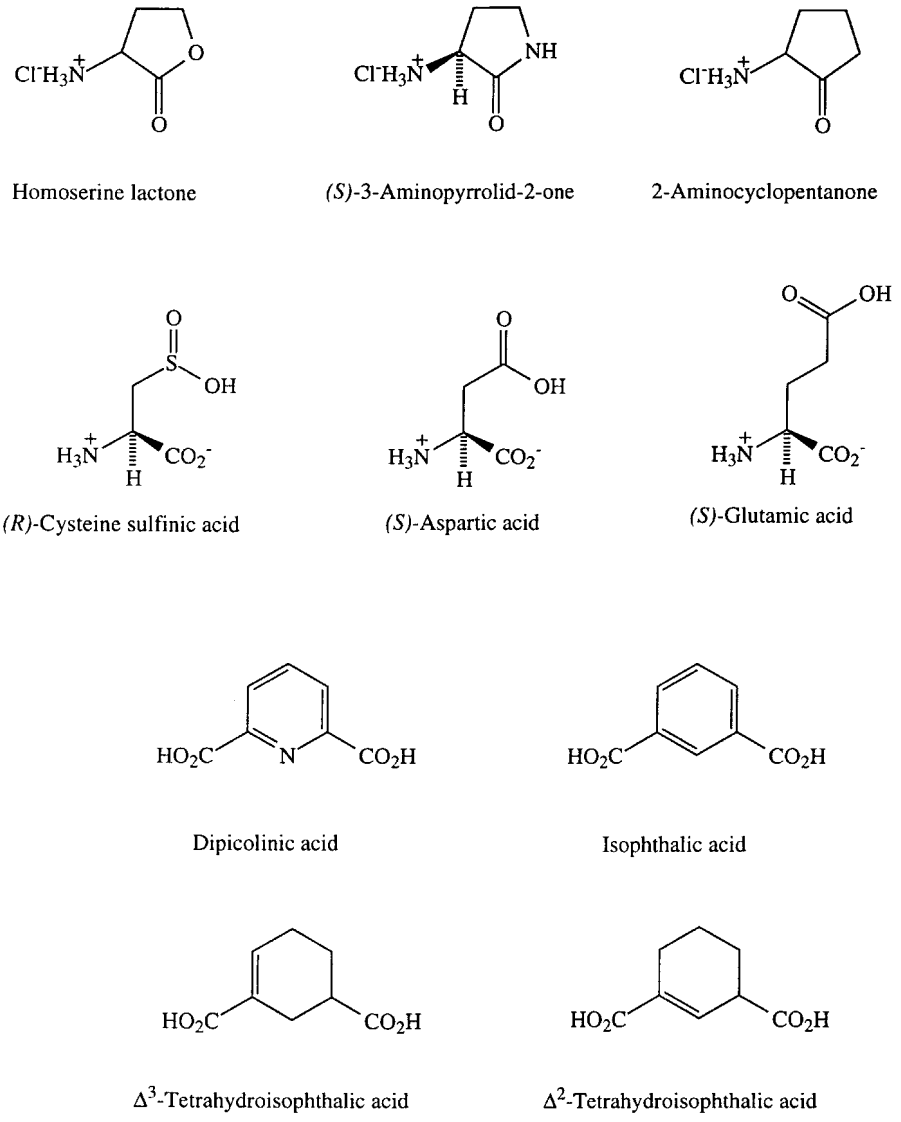

Figure 4. Inhibitors.

from the active site. This is consistent with the allosteric binding site for *(S)*-lysine that has been recently localised by X-ray crystallography.[5,8]

Kinetic studies were also performed to test analogues of the hypothesised cyclic lactol structure of *(S)*-ASA (Fig 2) for inhibition of DHDPS. These analogues included homoserine lactone, *(S)*-3-aminopyrrolid-2-one, and 2-aminocyclopentanone (Fig 4). Homoserine lactone was found to be a non-competitive inhibitor of DHDPS with respect to both substrates (Fig 5). Non-competitive inhibition was inconsistent with our hypothesis that homoserine lactone was a substrate mimic and suggests that homoserine lactone acts at an allosteric site, rather than at the active site. Inhibition by homoserine lactone may be consistent with its proposed role as a global starvation signal in *E coli*.[31]

Other cyclic analogues were also tested for their inhibitory properties. *(S)*-3-Aminopyrrolid-2-one did not inhibit DHDPS, whereas 2-aminocyclopentanone showed reversible, non-competitive inhibition with respect to *(S)*-ASA, with a higher K_i than that found for homoserine lactone. These molecules represent a new class of inhibitor for DHDPS and prospective lead compounds for herbicide research.

The results suggested that the biologically relevant form of *(S)*-ASA is indeed the straight chain hydrate (Fig 2).[9,10] Analogues of this hydrate were, therefore, tested as inhibitors of DHDPS (Fig 4). *(S)*-Asparagine, *S*-methyl-*(R)*-cysteine and the corresponding sulfoxide and sulfone, did not inhibit DHDPS. *(R)*-Cysteine sulfinic acid gave inhibition of DHDPS, which was uncompetitive with respect to *(S)*-ASA. *(S)*-Aspartic acid was a mixed-type inhibitor of DHDPS, with respect to *(S)*-ASA, (Fig 6). Finally, *(S)*-glutamic acid was an uncompetitive inhibitor of DHDPS, with respect to *(S)*-ASA.

Since none of these inhibitors simply competes with the substrate for the active site, we propose that they bind to the *(S)*-lysine feedback-inhibition site. One interpretation of the observation that *(S)*-aspartic acid is a mixed inhibitor (it has properties of both uncompetitive and competitive inhibition) is that the *(S)*-lysine feedback-inhibition allosteric site and the *(S)*-ASA active site may overlap and that *(S)*-aspartic acid binds there.

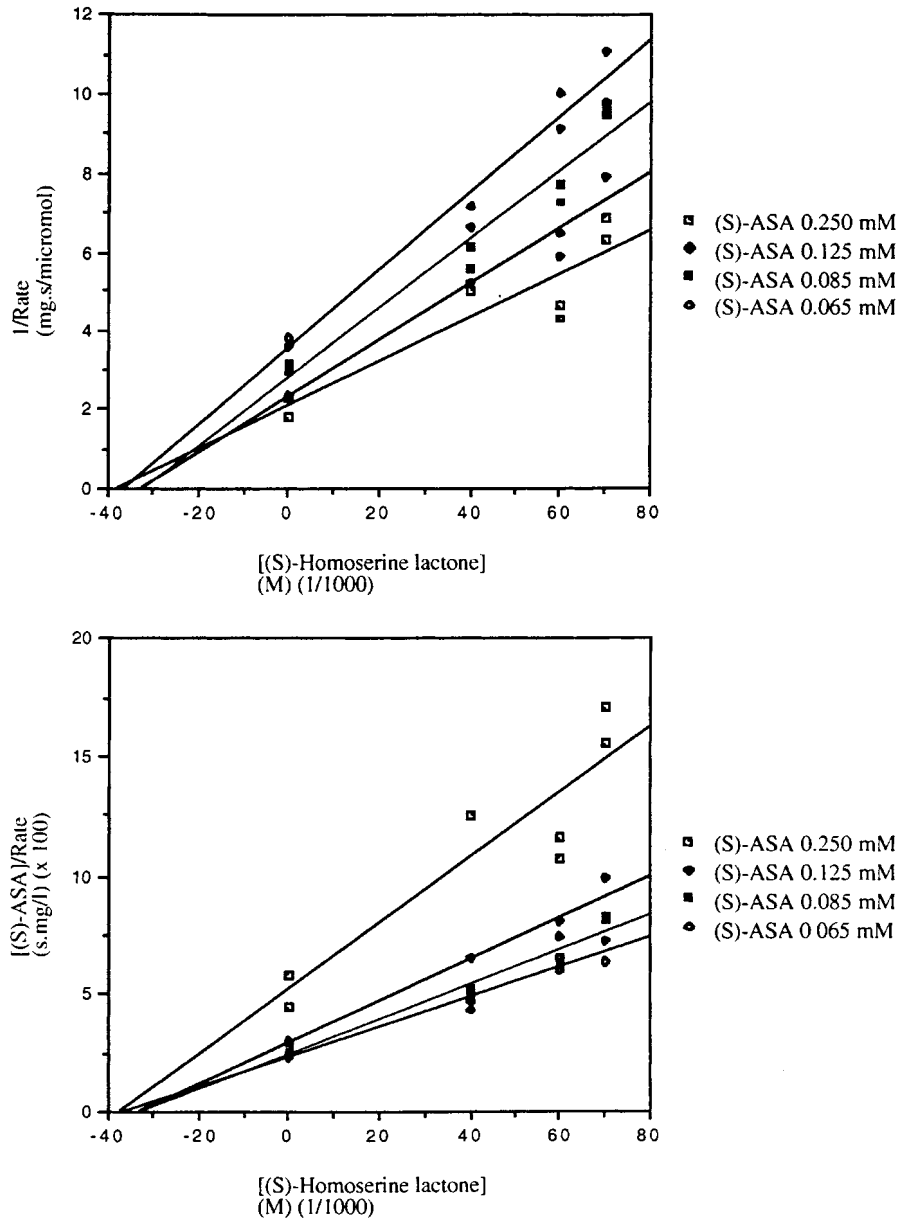

Figure 5. Dixon and modified Dixon plots of the inhibition of DHDPS by homoserine lactone with respect to (*S*)-ASA.

A variety of cyclic compounds (mainly aromatic) were tested as product inhibitors of DHDPS and substrate inhibitors of DHDPR. Of these, the best inhibitors of DHDPR were dipicolinic acid and isophthalic acid, (Fig 4). Dipicolinic acid was determined to be a competitive inhibitor of DHDPS with respect to the substrate, with a K_i value that fell between two literature values.[6,25] Similarly, isophthalic acid was a competitive inhibitor of DHDPS with respect to the substrate, with a K_i lower than the literature value.[25]

Finally, in an attempt to mimic 4-hydroxytetrahydrodipicolinic acid (HTHDPA) and 2,3,4,5-tetrahydropicolinate, isomers of tetrahydroisophthalic acid were synthesised and tested for inhibition of DHDPS and DHDPR. We predicted that Δ^2-tetrahydroisophthalic acid would be a stronger inhibitor of DHDPS and DHDPR than the Δ^3-tetrahydroisophthalic acid isomer, since the double bond in the Δ^2 position more closely resembles HTHDPA and 2,3,4,5-tetrahydrodipicolinate. However, Δ^2-tetrahydroisophthalic acid did not show potent inhibition of DHDPS or DHDPR, whereas Δ^3-tetrahydroisophthalic acid was an inhibitor of DHDPS (IC_{50} 1.5×10^{-2}M) and a stronger inhibitor of DHDPR (IC_{50} 4×10^{-3}M). This may suggest that the physiologically relevant form of this molecule is the enamine (Fig 7), where the double bond is in the Δ^3 position. This is consistent with the reported equilibration of these species *in vitro*.[32]

4 CONCLUSION

A coupled assay for DHDPS and DHDPR has proved ideal for detailed kinetic studies of both enzymes. Using this assay, several classes of inhibitor have been tested and their exact mode of inhibition determined.

A new class of DHDPS inhibitor has been identified, which includes homoserine lactone and

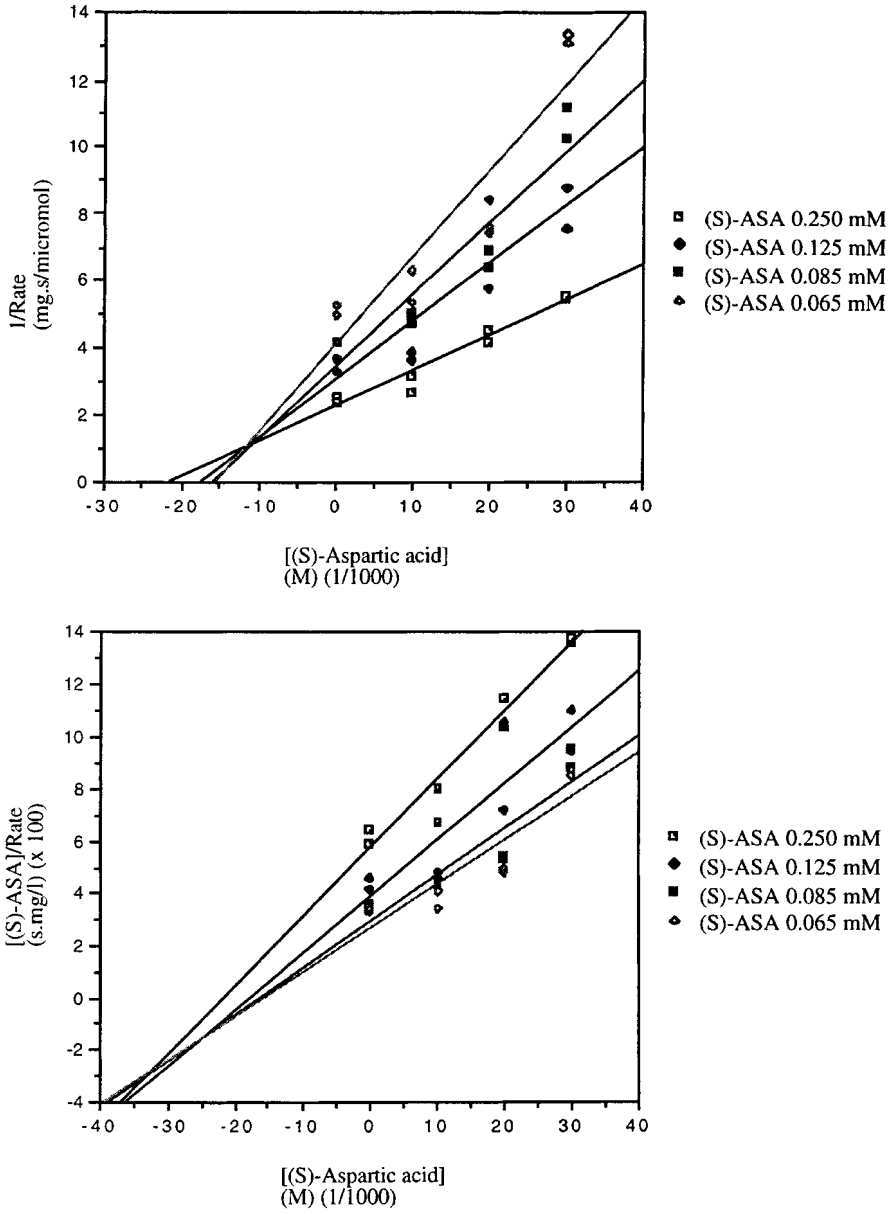

Figure 6. Dixon and modified Dixon plots of the inhibition of DHDPS by (*S*)-aspartic acid with respect to (*S*)-ASA.

2-aminocyclopentanone. These molecules show non-competitive inhibition with respect to both substrates, which we believe may reflect the role of homoserine lactone as a starvation signal *in vivo*.[31] DHDPS was found to be feedback-inhibited by *(S)*-lysine, where the *(S)*-lysine binds to an allosteric site. A substrate mimic that shows simple competitive inhibition with the *(S)*-ASA has not yet been found. *(R)*-Cysteine sulfinic acid and *(S)*-glutamic acid were uncompetitive inhibitors of DHDPS and *(S)*-aspartic acid was a mixed-type inhibitor, showing competitive and uncompetitive effects simultaneously. It is suggested that these inhibitors may bind to the *(S)*-lysine allosteric site.

Dipicolinic acid and isophthalic acid were strong competitive inhibitors of DHDPR. Δ^3-Tetrahydroisophthalic acid was a moderate inhibitor of both DHDPS and DHDPR, whereas Δ^2-tetrahydroisophthalic acid showed virtually no inhibition. This suggests that the physiologically relevant form of tetrahydrodipicolinate may be the enamine. Further work is underway to test this hypothesis. We are also continuing to synthesise analogues of the inhibitors reported herein, as potential novel herbicides.

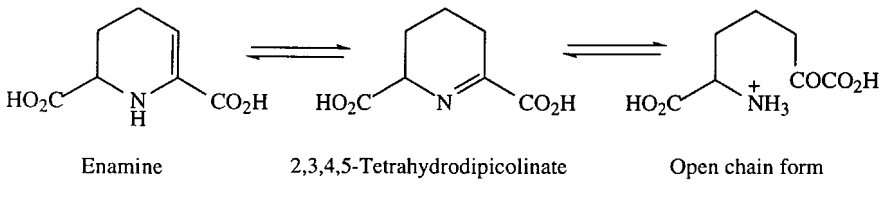

Figure 7. Equilibria of 2,3,4,5-tetrahydrodipicolinate.

ACKNOWLEDGEMENTS

Generous funding by Shell Research Limited made the initial stages of this work possible. We thank Carol Robinson for performing the electrospray mass spectrometry work.

REFERENCES

1 Cox RJ, The dap pathway to lysine as a target for antimicrobial agents. *Natural Product Reports* **13**:29–43 (1996).
2 Mazelis M, Whatley FR and Whatley J, The enzymology of lysine biosynthesis in higher plants. *FEBS Letts* **84**:236–240 (1977).
3 Bright SWJ and Shewry PR, Improvement of protein quality in cereals. *CRC Critical Reviews in Plant Science* **1**:49–93 (1983).
4 Bachmann B, Linkage map of *Escherichia coli* K-12, Edition 8. *Microbiol Rev* **54**:130–197 (1990).
5 Blickling S, Renner C, Laber B, Pohlenz H-D, Holak TA and Huber R, Reaction mechanism of *Escherichia coli* dihydrodipicolionate synthase investigated by X-ray crystallography and NMR spectroscopy. *Biochemistry* **36**:24–33 (1997).
6 Reddy SG, Sacchettini JC and Blanchard JS, Expression, purification, and characterisation of *Escherichia coli* dihydrodipicolinate reductase. *Biochemistry* **34**:3492–3501 (1995).
7 Patte J-C, Biosynthesis of threonine and lysine, in *Escherichia and Salmonella Cellular and Molecular Biology*, 2nd edn, Ed by Neidhardt FC, ASM Press, Washington. pp 528–541 (1996).
8 Blickling S and Knablein J, Feedback inhibition of dihydrodipicolinate synthase enzymes by L-lysine. *Biol Chem* **378**:207–210 (1997).
9 Coulter CV, Gerrard JA, Kraunsoe JAE, Moore DJ and Pratt AJ, *(S)*-Aspartate semi-aldehyde: synthetic and structural studies. *Tetrahedron* **52**:7127–7136 (1996).
10 Tudor DW, Lewis T and Robins DJ, Synthesis of the trifluoroacetate salt of aspartic acid β-semialdehyde, and intermediate in the biosynthesis of L-lysine, L-threonine, and L-methionine. *Synthesis* 1061–1062 (1993).
11 Smith LI and Opie JW, *o*-Aminobenzaldehyde. *Organic Synthesis* Coll Vol **III**. pp 56–58 (1995).
12 Wilkinson S, $\alpha\gamma$-Diaminobutyric acid. *J Chem Soc* 104–108 (1951).
13 Baumgarten HE and Peterson JM, Phenacylamine hydrochloride. *Organic Synthesis* Coll Vol **IV**. pp 909–917 (1973).
14 Johnson CR and Westrick MP, Sulfoxides. *Meth Enz* **143**:281–286 (1987).
15 Griffith OW, Amino acid sulfones: *S*-benzyl-DL-α-methyl sulfone. *Meth Enz* **143**:274–279 (1987).
16 Perkin WH and Pickles SS, The reduction of *iso*phthalic acid. *J Chem Soc* 293–313 (1905).
17 Kon GAR and Nandi BL, The structure of the glutaconic acids and esters. Part VIII. Some cyclic compounds of the glutaconic and the citraconic Acid series. *J Chem Soc* 1628–1634 (1933).
18 Dunn MJ, Initial planning, in *Protein Purification Methods*, Ed by Harris EL and Angal S, IRL at Oxford University Press, Oxford. pp 21–26 (1989).
19 Gerrard JA, Studies on dihydrodipicolinate synthase. D Phil Thesis, Brasenose College, Oxford (1992).
20 Shedlarski JG and Gilvarg C, The pyruvate-aspartic semialdehyde condensing enzyme of *Escherichia coli*. *J Biol Chem* **245**:1362–1373 (1970).
21 Kraunsoe JAE, Studies in lysine biosynthesis. D Phil Thesis, Corpus Christi College, Oxford (1992).
22 Yugari Y and Gilvarg C, The condensation step in diaminopimelate synthesis. *J Biol Chem* **240**:4710–4716 (1965).
23 Shedlarski JG, Pyruvate-aspartic semialdehyde condensing enzyme *(Escherichia coli)*. *Meth Enz* **17b**:129–134 (1971).
24 Laber B, Gomis-Ruth F-X, Romao MJ and Huber R, *Escherichia coli* dihydrodipicolinate synthase. *Biochem J* **288**:691–695 (1992).
25 Tamir H and Gilvarg C, Dihydrodipicolinic acid reductase. *J Biol Chem* **249**:3034–3040 (1974).
26 Farkas W and Gilvarg C, The reduction step in diaminopimelic acid biosynthesis. *J Biol Chem* **240**:4717–4722 (1965).
27 Reddy SG, Sacchettini JC and Blanchard JS, Expression, purification, and characterisation of *Escherichia coli* dihydrodipicolinate reductase. *Biochemistry* **34**:3492–3501 (1995).
28 Karsten WE, Dihydrodipicolinate synthase from *Escherichia coli:* pH-dependent changes in the kinetic mechanism and kinetic mechanism of allosteric inhibition by L-lysine. *Biochemistry* **36**:1730–1739 (1997).
29 Couper L, McKendrick JE and Robins DJ, Pyridine and piperidine derivatives as inhibitors of dihydrodipicolinic acid synthase, a key enzyme in the diaminopimelate pathway to L-lysine. *Bioorg Med Chem Letts* **4**:2267–2272 (1994).
30 Cornish-Bowden A, A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem J* **137**:143–144 (1974).
31 Huisman GW and Kolter R, Sensing starvation: A homoserine lactone-dependent signalling pathway in *Escherichia coli*. *Science (Washington)* **265**:537–539 (1994).
32 Chrystal EJT, Couper L and Robins DJ, Synthesis of a key intermediate in the diaminopimelate pathway to L-lysine: 2,3,4,5-tetrahydrodipicolinic acid. *Tetrahedron* **51**:10241–10252 (1995).